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Metabolic interconnectivity among alternative respiration, residual respiration, carbohydrates and phenolics in leaves of *Salvinia minima* exposed to Cr(VI)

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ABSTRACT

Floating and submerged leaves of the aquatic fern *Salvinia minima* were used to analyze a metabolic interconnectivity among mitochondrial alternative respiration, residual respiration (R_{resp}), carbohydrate metabolism and soluble phenolics (SP) accumulation occurring under Cr(VI) stress. Treatment with Cr enhanced alternative pathway capacity (AP_{cap}) and (R_{resp}) in both leaf types. AP_{cap}/T_{resp} ratio revealed an increasing relative contribution of the alternative respiration to total respiration rate under Cr(VI) treatment. Sucrose content increased in Cr-treated leaves, but glucose and starch decreased. Enzyme profile showed that sucrose synthase (SS) rather than soluble acid invertase (AI) seems to be involved in sucrose metabolism of Cr-treated plants. Accumulation of SP showed a positive correlation with both AP_{cap} and R_{resp} in floating leaves. Decreases of SP in submerged leaves can be explained by an increased synthesis of polymerized phenolics. Results provide important new insights about influence of alternative and residual respirations on the synthesis of phenylpropanoid-derivative compounds. This work could also represent the first communication about involvement of the R_{resp} in defence mechanism of *S. minima* against Cr(VI) toxicity.

1. Introduction

Mitochondrial respiration in plants plays a key role in the central metabolism and contributes in different ways to cell function. It is a source of ATP and intermediate metabolites to many synthetic processes (Plaxton and Podesta, 2006). Respiration plays a special role in plant adaptation to environmental adverse conditions i.e. abiotic and biotic stresses. Plants exposed to abiotic stress such as heavy metals exhibit decreased respiratory activity and less metabolic performance. At molecular level, oxidative stress is widely recognized as a key sign of heavy metal-stressed plants (Lösch and Köhl, 1999). Oxidative stress arises from an imbalance between the generation of reactive oxygen species (ROS) and antioxidants in favour of the former (Møller, 2001). Although, mitochondrial respiration is a ubiquitous process in all plant cells providing most of the cellular energy in heterotrophic tissues, in photosynthetically active tissues this situation is somewhat different with photosynthesis playing a major role in this process and a further pathway, photorespiration, exerting great influence on cell energy balance. Respiration and photosynthesis are interdependent processes with the former relying on photosynthesis for substrate, and the latter depending on respiration for a range of compounds such as ATP and reducing equivalents (Hoefnagel et al., 1998).

Mitochondria in higher plants exhibit a branch point in the respiratory electron transport chain after ubiquinone. One channelizes electrons through the cytochrome *c* pathway (CP) that generates the proton-motive force used for ATP synthesis, and the other is an alternative, non-phosphorylating, pathway (AP) which through an alternative oxidase (AOX) that is cyanide insensitive, channelizes electrons directly from ubiquinone to O_2 (van Dongen et al., 2011). Since, AP is uncoupled from proton translocation and thereby ATP synthesis, operation of this pathway apparently wastes energy. However, AP is thought to function as an antioxidant system to suppress the generation of ROS (Maxwell et al., 1999). Several positive roles for AP in plant metabolism have been suggested. It may be up-regulated under stress conditions to act as sink for reducing power, preventing accumulation of the excess reducing equivalents in chloroplasts and thereby decreasing the probability of loss of the photosynthetic function. Moreover, it may allow carbon flux through the tricarboxylic acid (TCA) cycle when ADP supply limits the activity of CP, then providing carbon skeletons for other cellular processes (Borecký and Vercesi, 2005; Gandin et al., 2009). It has also been suggested that AP plays an important role to regulate redox status and related metabolic pathways in non-photosynthetic tissues (van Dongen et al., 2011). When CP is inhibited, NADH/NAD+ ratio increases and thus TCA cycle slows down, giving a considerably lower amount of carbon skeletons

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available for metabolic pathways that are directly derived from TCA cycle intermediates. In these conditions, AP modulates the reduction state of electron transport chain components, and hence the rate of generation of ROS. Indeed, ROS production by mitochondrial electron chain induces the expression of AOX genes (Gupta et al., 2009). Additionally, it has also been hypothesized that AP plays a key role in the maintenance of cellular oxygen homeostasis. According with this hypothesis, when oxygen concentration is high, AP can reduce the excess of oxygen, without limiting oxygen supply to CP, minimizing, at the same time, mitochondrial ROS production (Gupta et al., 2009). In fact, most of roles postulated for AP can be summarized as that it serves to counteract deleterious short-term metabolic fluctuations, especially under stress conditions, that would otherwise lead to oxidative stress (Rasmusson et al., 2009). Nevertheless, several evidences indicated that AP plays a key role in adjusting mechanisms of plants to unfavourable environmental conditions including heavy metal pollution (Castro-Guerrero et al., 2008; Van Aken et al., 2009; Hanqing et al., 2010). It is noteworthy that often even in the presence of both cyanide and SHAM, O_2 uptake is not always totally inhibited. This component of O_2 uptake has been called residual respiration (R_{resp}) (Ribas-Carbo et al., 1997). Although R_{resp} is known for many years its origin still remains unclear. In a recent work on this enigmatic respiration was assumed that an AOX-lacking leaf might induce a residual O_2 consuming process that would not normally be seen in an AOX-containing leaf (Guy and Vanlerberghe, 2005). However, it is unlikely that R_{resp} is mitochondrial in nature due to it only appears when using intact tissues, not isolated mitochondria (Atkin et al., 2002). Whether residual respiration occurs in the absence of SHAM and cyanide is unknown. Exact role of R_{resp} was not clarified yet, but there are some evidences linking it to stress conditions such as drought and mineral deficiency (Rakhmankulova et al., 2003; Shugaeva et al., 2007).

Because carbohydrate metabolism and respiration are coupled processes, there must be mechanisms to integrate them for changes in the supply of – or demand for – carbon skeletons, reducing power and ATP to support both catabolic and anabolic pathways occurring in heavy metal-stressed plants. However, how different respiratory pathways are involved in the tolerance of plants to heavy metals in relation to metal-induced oxidative stress and synthesis of secondary metabolites remains still largely unknown. Thus, the goal of this study was to verify the hypothesis that both alternative and residual respiration linked to carbohydrate metabolism and peroxidase (POD) activity, contribute to increase the synthesis of secondary metabolites in photosynthetic and non-photosynthetic tissues of Cr-stressed plants. Based on this hypothesis a thorough analysis of the effects of Cr(VI) on floating and submerged leaves of the aquatic fern *Salvinia minima* was undertaken in this work, including evaluation of changes occurring in total respiration (T_{resp}), cytochrome pathway capacity (CP_{cap}), alternative pathway capacity (AP_{cap}), residual respiration (R_{resp}), carbohydrates, sucrose-related enzymes, soluble phenolics (SP) and guaiacol-peroxidase activity (G-POD).

2. Materials and methods

2.1. Plant material

S. minima were collected according to uniform size from a non-polluted artificial pond located in the Campus of the School of Natural Sciences (26°50′N, 65°12′W, Tucumán, Argentina). The study was conducted using 7-day-old floating and submerged leaves from plants grown in polycarbonate pots filled either with tap water or Cr(VI) solution under controlled conditions (14-h light period, 25/20 °C day/night temperature, 60% relative humidity, and 190 μmol m^{-2} s^{-1}). We chose 7 days as experimental period because preliminary experiments carried out during 9 days showed slightly symptoms of chlorosis and necrosis in Cr-treated plants. Cr(VI) solution was prepared using tap water in order to get a similar aquatic medium than the pond where plants were collected. Plants were not cultivated in Hoagland's mineral solution to avoid ionic interactions and/or ion competition between Hoagland salts and dichromate salt. Cr(VI) was provided as analytical grade potassium dichromate ($K_2Cr_2O_7$). A concentrated solution (500 mg L^{-1}) was prepared, and then different dilutions (0, 2, 5 and 10 mg L^{-1}) were prepared using tap water. Because Cr(VI) remotion from water by aquatic macrophytes is a fast process (Maine et al., 2004), the Cr solution was renewed at the middle of the experimental period to avoid excessive changes in Cr(VI) concentration. The pH of Cr solution and control tap water ranged between 6.8 and 7.0 during the experimental period and it was daily measured using a glass combination pH-sensitive electrode coupled to a digital pH meter (Hanna Instrument, Germany). At this pH value, spontaneous reduction of Cr(VI) to Cr(III) was not detected. In order to minimize any diurnal effect on respiration and chemical determinations leaf samples were collected at noon. Wet plant biomass (FW) was immediately determined after harvesting whereas the dry plant biomass (DW) was determined by drying weighed wet samples at 80 °C in a hot air oven for 4 days and weighed again.

2.2. Respiration measurements

Total respiration (T_{resp}), and cytochrome (CP) and alternative (AP) respiratory pathways were measured polarographically with a Clark electrode (Yellow Springs Instrument Co. USA) at 28 °C in a thermostatted cell, and recorded in a Gilson oxygraph (Gilson Medical Electronics, Inc. USA). Floating and submerged leaves from both Cr-untreated (control) and Cr-treated plants were harvested in the seventh day and sliced with a razor blade at approximately 1 mm strips to yield 70 and 100 mg FW of floating and submerged leaves, respectively. Strips were submerged in 2 mL of an air saturated solution (initial concentration of O_2 was considered to be 240 μM) containing 50 mM NaH_2PO_4/Na_2HPO_4 buffer (pH 7.0). This pH value was selected because it is similar to pH of the artificial pond. Incubation solution was constantly stirring with a micro stir bar. Oxygen consumption was measured using 1 mM potassium cyanide (KCN) as inhibitor of CP and 3 mM salicylhydroxamic acid (SHAM) as inhibitor of AP. Optimal concentrations of inhibitors, sufficient for full inhibition of the respective pathway without apparent side effects in our system, were revealed by titration curves in preliminary experiments using different concentrations of each inhibitor in presence and absence of the other. Steady rates of respiratory O_2 consumption were determined after about 20 min under assay conditions. T_{resp} was measured in absence of inhibitors. Respiratory cytochrome pathway capacity (CP_{cap}) and alternative pathway capacity (AP_{cap}) were determined by subtracting residual respiration (R_{resp}) either from the SHAM-sensitive respiration or from cyanide-sensitive respiration, respectively (Vanlerberghe and McIntosh, 1992). R_{resp} was determined from the rate of O_2 consumption in presence of both inhibitors (KCN + SHAM). AP_{cap}/T_{resp} ratio was also calculated. Respiratory O_2 consumption was expressed as nmol O_2 mg^{-1} FW min^{-1}.

2.3. Soluble carbohydrates and starch

Soluble carbohydrates (sucrose, glucose and fructose) were extracted and quantified from floating and submerged leaves as described by Prado et al. (2010). Sugar content was expressed as mg g^{-1} FW. After soluble carbohydrates extraction, remaining insoluble starch was determined measuring the release of reducing

sugars after enzymatic starch hydrolysis (Rosa et al., 2009). Starch content was expressed as mg maltose equivalent g^{-1} FW.

2.4. Sucrose-related enzymes (SPS, SS, AI)

Activities of soluble acid invertase (AI), sucrose synthase (SS) and sucrose phosphate synthase (SPS) were measured in floating and submerged leaves as described by Rosa et al. (2009). Briefly, leaf samples (1.0 g FW) were homogenized in a chilled mortar and pestle either with 3 mL 50 mM sodium phosphate buffer (pH 7.4), containing 1 mM β-mercaptoethanol and 5 μM $MnSO_4$ (AI), or 100 mM Tris–HCl buffer (pH 7.6), containing 10 mM $MgCl_2$, 5 mM EDTA, and 1 mM β-mercaptoethanol (SS and SPS). Resulting homogenates were centrifuged at 12,000 × *g* for 15 min at 4 °C, and supernatants were dialyzed against 10 mM sodium acetate buffer (pH 5.5), containing 1 mM β-mercaptoethanol (AI) and 10 mM Tris–HCl buffer (pH 8.0), containing 10 mM $MgCl_2$, and 1 mM β-mercaptoethanol (SS and SPS). Dialyzed supernatants were used as enzyme extracts. AI activity was assayed as described in Prado et al. (1985), whereas SS and SPS activities were assayed according to Rosa et al. (2009). SS and SPS activities were assayed following formation of sucrose and sucrose-6-P, respectively. Enzyme activities were expressed as μmol product g^{-1} protein min^{-1}.

2.5. Soluble guaiacol-peroxidase (G-POD)

G-POD activity was extracted by homogenizing leaf samples (1 g FW) in 3 mL 25 mM Tris–HCl buffer (pH 7.2), containing 5 mM cysteine and 5 mM EDTA. After centrifugation at 12,000 × *g* for 20 min at 4 °C, supernatant was used as enzyme extract. To measure G-POD activity an aliquot of the supernatant (200 μL) was added to 2 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 6.0), 1% guaiacol and 0.4% H_2O_2. Oxidation of guaiacol was measured at 420 nm against a blank without H_2O_2 (Dhir et al., 2009). G-POD activity was expressed as ΔA_{420} g^{-1} protein min^{-1}.

2.6. Soluble phenolics (SP)

SP were extracted with ethanol 96% and determined by using the Folin–Ciocalteu's reagent (Swain and Hillis, 1959). Briefly, leaf samples (1.0 g FW) were extracted with 3 mL 96% ethanol incubated in darkness at room temperature for 48 h and centrifuged a 3000 × *g* for 5 min. An aliquot supernatant (0.1 mL) was added with 0.2 mL (1:1, v/v) diluted Folin–Ciocalteu reagent and 1.8 mL distilled water. After incubation at room temperature for 2 min the reaction mixture was added with 0.8 mL 7.5% Na_2CO_3 and incubated at room temperature for another 5 min. Absorbance was read at 760 nm. SP concentration was determined using a calibration curve made with pure phenol and expressed as μmol phenol equivalent g^{-1} FW.

2.7. Protein and Cr(VI) accumulation

Protein content of floating and submerged leaves was determined according to Lowry et al. (1951) using bovine serum albumin (BSA) as standard. Analytical method and data on Cr(VI) accumulation have been reported previously (Prado et al., 2010).

2.8. Statistical

Results are means of three independent replicates. Data were processed with analysis of variance (ANOVA) and means were compared using Tukey's test at 5% level ($p < 0.05$).

3. Results

3.1. Respiration measurements

Table 1 presents the results of respiration measurements in both floating and submerged leaves of *Salvinia* plants grown in presence of different Cr(VI) concentrations. In general, respiration measurements were higher in floating leaves than in submerged ones. In floating leaves T_{resp} increased from 1.54 nmol O_2 mg^{-1} FW min^{-1} in Cr-untreated leaves to 2.61 nmol O_2 mg^{-1} FW min^{-1} under 5 mg L^{-1} Cr(VI) concentration, and then declined to 1.82 nmol O_2 mg^{-1} FW min^{-1} at 10 mg L^{-1} Cr(VI) concentration. AP_{cap} showed a similar activity pattern, but the increase of O_2 consumption was more pronounced. Values increased from 0.097 (control) to 0.339 nmol O_2 mg^{-1} FW min^{-1} [5 mg L^{-1} Cr(VI)], and then decreased until 0.181 nmol O_2 mg^{-1} FW min^{-1} at the highest Cr(VI) concentration. Similarly, CP_{cap} increased from 1.38 nmol O_2 mg^{-1} FW min^{-1} (Cr-untreated) to 2.07 nmol O_2 mg^{-1} FW min^{-1} at 5 mg L^{-1} Cr(VI) concentration, and then decreased until 1.48 nmol O_2 mg^{-1} FW min^{-1} under highest Cr(VI) concentration. At all Cr(VI) concentrations, AP_{cap} was significantly higher ($p < 0.05$) in Cr-treated than in Cr-untreated leaves, while T_{resp} and CP_{cap} showed a significant increase at 5 mg L^{-1} Cr(VI) concentration only. Highest values of T_{resp}, CP_{cap} and AP_{cap} were 69.5%, 50.0% and 3.5-fold higher than control values. In submerged leaves, T_{resp}, CP_{cap} and AP_{cap} showed significant initial increases ($p < 0.05$) up to 2 mg L^{-1} Cr(VI) concentration and then stayed approximately constants until the end of experiment. Highest values of T_{resp} and CP_{cap} were observed at 2 mg L^{-1} Cr(VI) concentration (0.86 and 0.76 nmol O_2 mg^{-1} FW min^{-1}, respectively), while highest value of AP_{cap} (0.045 nmol O_2 mg^{-1} FW min^{-1}) was found at 10 mg L^{-1} Cr(VI) concentration. These values were 65.4%, 68.9% and 5-fold higher than corresponding control values. It is necessary to point out that AP_{cap} values do not reflect actual AOX activity during respiration in uninhibited conditions, because the addition of inhibitors may modify partitioning of electron flux over CP and AP pathways (Millar et al., 1995). Thus, AP_{cap} shows activity only under given set of conditions used. Values of AP_{cap}/T_{resp} ratio, a measure of the relative contribution of AP to T_{resp} (Feng et al., 2008), ranged from 6.5% (Cr-untreated) to 13.0% [5 mg L^{-1} Cr(VI)] in floating leaves and from 1.7% (control) to 6.2% [10 mg L^{-1} Cr(VI)] in submerged leaves. R_{resp} also enhanced under Cr(VI) exposure being significantly higher ($p < 0.05$) at 5 and 10 mg L^{-1} Cr(VI) concentration in both leaf types. In floating leaves, R_{resp} rate increased from 0.012 nmol O_2 mg^{-1} FW min^{-1} in Cr-untreated control to 0.070 nmol O_2 mg^{-1} FW min^{-1} at 10 mg L^{-1} Cr(VI) concentration. In submerged leaves minimum and maximum values were 0.016 nmol O_2 mg^{-1} FW min^{-1} (Cr-untreated leaves) and 0.054 nmol O_2 mg^{-1} FW min^{-1} at highest Cr(VI) concentration used. Participation of R_{resp} in total respiration rate ranged between 0.8% (control) and 3.8% (Cr-treated) in floating leaves and from 3.1% (control) to 7.5% (Cr-treated) in submerged leaves, respectively (data not shown).

3.2. Soluble carbohydrates and starch

Treatment with Cr(VI) caused changes in sucrose, glucose, fructose and starch contents of *Salvinia* leaves. Results showed that each sugar exhibited a similar distribution pattern in both leaves, but highest values were always found in floating leaves (Table 2). In floating leaves, sucrose and fructose concentrations increased with increasing Cr(VI), reaching maxima values of 0.072 and 0.128 mg g^{-1} FW, at 10 and 5 mg L^{-1} Cr(VI) concentrations, respectively. These values were 6.5-fold and 50.6% higher than those observed in Cr-untreated leaves. In submerged leaves maxima

Table 1
Total respiration (T_{resp}), cytochrome pathway capacity (CP_{cap}), alternative pathway capacity (AP_{cap}), residual respiration (R_{resp}) and alternative pathway capacity/total respiration rate (AP_{cap}/T_{resp}) of floating and submerged leaves of *S. minima* exposed to increasing concentrations of Cr(VI) during 7 days. Values followed by the same letter within a column are not significantly different at $p < 0.05$ ($n = 6$). Lowercase letters are used to denote significance among leaves of the floating leaves values. Uppercase letters are used to denote significance among values of the submerged leaves.

Cr(VI) (mg L^{-1})	T_{resp}	CP_{cap}	AP_{cap} (nmol O_2 mg^{-1} FW min^{-1})	R_{resp}	AP_{cap}/T_{resp} (%)
Floating leaves					
0	1.54 ± 0.18[a]	1.38 ± 0.13[a]	0.097 ± 0.011[a]	0.012 ± 0.002[a]	6.5
2	1.69 ± 0.15[a]	1.48 ± 0.15[a]	0.137 ± 0.009[b]	0.014 ± 0.001[a]	8.3
5	2.61 ± 0.16[b]	2.07 ± 119[b]	0.339 ± 0.031[c]	0.064 ± 0.007[b]	13.0
10	1.82 ± 0.18[a]	1.48 ± 0.15[a]	0.181 ± 0.014[d]	0.070 ± 0.010[b]	9.9
Submerged leaves					
0	0.52 ± 0.04[A]	0.45 ± 0.03[A]	0.009 ± 0.001[A]	0.016 ± 0.002[A]	1.7
2	0.86 ± 0.06[B]	0.76 ± 0.05[B]	0.041 ± 0.006[B]	0.017 ± 0.001[A]	4.8
5	0.81 ± 0.06[B]	0.73 ± 0.07[B]	0.044 ± 0.004[B]	0.044 ± 0.005[B]	5.4
10	0.72 ± 0.07[B]	0.66 ± 0.05[B]	0.045 ± 0.005[B]	0.054 ± 0.003[C]	6.2

Table 2
Concentrations of soluble sugars (sucrose, glucose and fructose) and starch in floating and submerged leaves of *S. minima* growing under increasing Cr(VI) concentrations during 7 days. Values followed by the same letter within a column are not significantly different at $p < 0.05$ ($n = 6$). Lowercase letters are used to denote significance among leaves of the floating leaves values. Uppercase letters are used to denote significance among values of the submerged leaves.

Cr(VI) (mg L^{-1})	Sucrose (mg g^{-1} FW)	Glucose (mg g^{-1} FW)	Fructose (mg g^{-1} FW)	Starch (mg mal. g^{-1} FW)
Floating leaves				
0	0.011 ± 0.001[a]	0.111 ± 0.012[a]	0.085 ± 0.006[a]	1.24 ± 0.12[a]
2	0.037 ± 0.002[b]	0.069 ± 0.005[b]	0.075 ± 0.007[a]	1.14 ± 0.14[a]
5	0.063 ± 0.005[c]	0.074 ± 0.006[b]	0.128 ± 0.011[b]	0.94 ± 0.09[a]
10	0.072 ± 0.006[c]	0.060 ± 0.005[c]	0.109 ± 0.009[b]	1.09 ± 0.13[a]
Submerged leaves				
0	0.011 ± 0.001[A]	0.061 ± 0.005[A]	0.010 ± 0.001[A]	0.59 ± 0.07[A]
2	0.021 ± 0.002[B]	0.048 ± 0.005[B]	0.011 ± 0.001[A]	0.37 ± 0.03[B]
5	0.044 ± 0.002[C]	0.013 ± 0.002[C]	0.030 ± 0.001[B]	0.33 ± 0.03[B]
10	0.055 ± 0.004[D]	0.015 ± 0.002[C]	0.006 ± 0.001[C]	0.37 ± 0.02[B]

values for sucrose and fructose were 0.055 and 0.03 mg g^{-1} FW and were found at the same Cr(VI) concentrations. These concentrations were 5- and 3-fold higher than in control leaves. Of interest, at 10 mg L^{-1} Cr(VI) concentration the content of fructose was lower than that at 5 mg L^{-1} metal concentration in both leaf types. Glucose concentration was decreased significantly ($p < 0.05$) in floating and submerged leaves by Cr(VI) treatment when comparing with Cr-untreated leaves. Minima concentrations, 0.06 and 0.013 mg g^{-1} FW, were found under 10 and 5 mg L^{-1} Cr(VI) concentrations in floating and submerged leaves, respectively. These concentrations were 85% and 4.7-fold lower than in Cr-untreated leaves. Although starch content also decreased in Cr-treated leaves, this was only significant in submerged leaves ($p < 0.05$). Maximum decrease, 78.8%, was observed at 5 mg L^{-1} Cr(VI) concentration (Table 2).

3.3. Sucrose-related enzymes (SPS, SS, AI)

Activities of sucrose-related enzymes i.e. SS, SPS and AI are depicted in Table 3. All enzyme activities were differently affected by Cr(VI) treatment even at lowest tested concentration (2 mg L^{-1}). SS and SPS activities were, in general, higher in submerged leaves than in floating leaves, while AI activity was higher in the latter. In submerged leaves , SPS and SS activities were higher in Cr-treated plants than in Cr-untreated plants. SPS activity pattern in floating leaves showed a slow initial decrease up to 2 mg L^{-1} Cr(VI) concentration followed by a progressive increase up to 10 mg L^{-1} metal concentration reaching a maximum value of 0.025 μmol suc-6-P g^{-1} protein min^{-1}. This activity was 66.7% higher than that found in Cr-untreated control. In submerged leaves, SPS activity was slightly reduced up to 2 mg L^{-1} Cr(VI) concentration and then it was strongly decreased. Lowest activity (0.042 μmol suc-6-P g^{-1} protein min^{-1}) was observed at 5 mg L^{-1} Cr(VI) concentration, being 83.3% lower than that Cr-untreated activity. SS activity increased strongly from Cr-untreated floating leaves up to the end of experiment, reaching a maximum value of 0.072 μmol sucrose g^{-1} protein min^{-1} at 10 mg L^{-1} Cr(VI) concentration, that was 4.2-fold higher than that control activity. In submerged leaves was found an initial increase of SS activity, reaching a value of 0.123 μmol sucrose g^{-1} protein min^{-1} at 2 mg L^{-1} Cr(VI) concentration, and then declined slightly up to 5 mg L^{-1} metal concentration. From this point on, SS activity decreased strongly reaching a minimum value of 0.041 μmol sucrose g^{-1} protein min^{-1}. Interestingly, this minimum activity was also higher than that control activity. SS activity in Cr-treated submerged leaves was 5.6-fold higher than that observed in Cr-untreated leaves. Cr treatment, excepting for 5 mg L^{-1} Cr(VI) concentration, decreased AI activity in floating leaves. Lowest activity (0.064 μmol red. sugar g^{-1} protein min^{-1}) was found at 10 mg L^{-1} Cr(VI) concentration, being 51.6% lower than that activity observed in Cr-untreated leaves. Contrarily, in submerged leaves AI activity decreased strongly under Cr(VI) exposure, giving a reduction of 8-fold in enzyme activity at 10 mg L^{-1} metal concentration. AI/SS ratio decreased in Cr-treated leaves, being more pronounced in submerged leaves when compared with controls ones (data not shown).

3.4. Soluble guaiacol-peroxidase (G-POD)

Excepting for floating leaves under 2 mg L^{-1} Cr(VI) concentration, activity of G-POD was increased significantly in both leaf types under Cr(VI) treatment. Although G-POD activity, in general, seemed to be higher in submerged leaves, statistical analysis did not revealed significant differences between leaves (Fig. 1). Highest values of G-POD in floating and submerged leaves were 516

Table 3
Activities of sucrose synthase (SS), soluble acid invertase (AI) and sucrose phosphate synthase (SPS) in floating and submerged leaves of *S. minima* exposed to different Cr(VI) concentrations during 7 days. Values followed by the same letter within a column are not significantly different at $p < 0.05$ ($n = 6$). Lowercase letters are used to denote significance among leaves of the floating leaves values. Uppercase letters are used to denote significance among values of the submerged leaves.

Cr(VI) (mg L^{-1})	SS (μmol suc. g^{-1} prot. min^{-1})	AI (μmol red. sug. g^{-1} prot. min^{-1})	SPS (μmol suc-6-P. g^{-1} prot. min^{-1})
Floating leaves			
0	0.017 ± 0.001^{a}	0.097 ± 0.005^{a}	0.015 ± 0.001^{a}
2	0.044 ± 0.002^{b}	0.078 ± 0.003^{b}	0.011 ± 0.001^{b}
5	0.056 ± 0.003^{c}	0.103 ± 0.006^{a}	0.022 ± 0.002^{c}
10	0.072 ± 0.005^{d}	0.064 ± 0.003^{d}	0.025 ± 0.002^{c}
Submerged leaves			
0	0.022 ± 0.002^{A}	0.064 ± 0.005^{A}	0.077 ± 0.005^{A}
2	0.123 ± 0.008^{B}	0.031 ± 0.002^{B}	0.069 ± 0.005^{A}
5	0.114 ± 0.007^{B}	0.017 ± 0.002^{C}	0.042 ± 0.003^{B}
10	0.041 ± 0.003^{D}	0.008 ± 0.001^{D}	0.047 ± 0.002^{B}

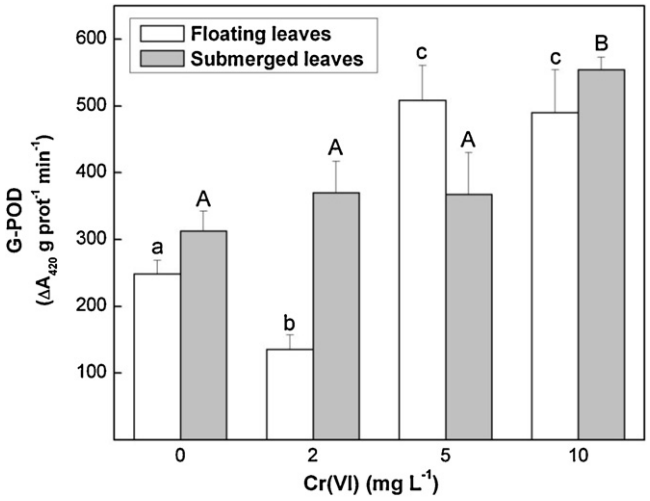

Fig. 1. Effect of Cr(VI) on G-POD activity in leaves of *S. minima* plants after 7 days of Cr treatment. Means of three independent experiments are represented. Bars on top indicate standard deviation at $p < 0.05$ ($n = 6$). Different letters on bars denote significant differences. Lowercase letters are used to denote significance among values of the floating leaves. Uppercase letters are used to denote significance among values of the submerged leaves.

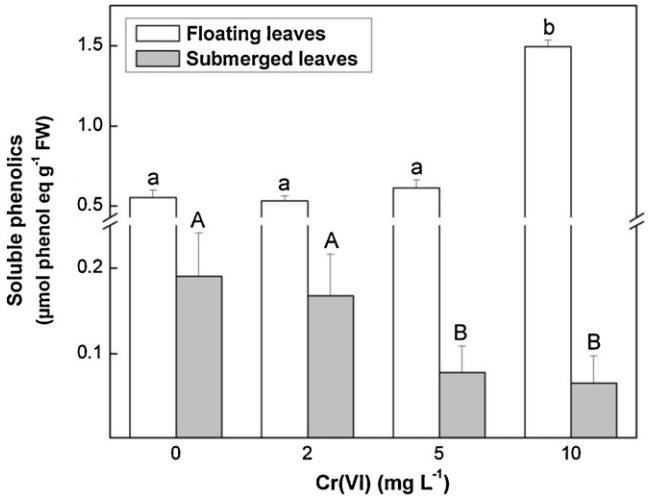

Fig. 2. Content of SP in leaves of *S. minima* after 7 days of Cr(VI) treatment. Means of three independent experiments are represented. Bars on top indicate standard deviation at $p < 0.05$ ($n = 6$). Different letters on bars denote significant differences. Lowercase letters are used to denote significance among values of the floating leaves. Uppercase letters are used to denote significance among values of the submerged leaves.

and 557 ΔA_{420} g protein^{-1} min^{-1}, which were 108% and 78% higher than values found in Cr-untreated leaves, respectively.

3.4.1. Soluble phenolics (SP)

Fig. 2 shows concentrations of SP in both floating and submerged leaves of *S. minima* grown in presence of different Cr(VI) concentrations. SP concentration was much higher in floating than in submerged leaves, mainly at highest Cr(VI) concentrations. In floating leaves SP strongly increased at 10 mg L^{-1} Cr(VI) concentration, giving an increase of 2.7-fold compared with Cr-untreated leaves. Contrarily, in submerged leaves a sustained decrease in the concentration of soluble phenolics was observed under Cr exposure.

4. Discussion

During plant growth, production of ROS by functioning mitochondrial and chloroplastic electron transport chains occurs normally (Dinakar et al., 2010). In heavy metal-stressed plants, however, metal may interact with different sites of both chains, allowing that certain reduced electron transport components become ROS generators (Dietz et al., 1999). Interactions of Cr(VI) on mitochondrial electron chain accompanied by enhanced production of ROS have been reported in several plants (Dixit et al., 2002; Shanker et al., 2005; Jasso-Chávez et al., 2010). But, it is not entirely clear as Cr-induced oxidative stress affects both carbon partitioning and respiratory energy efficiency, and how these processes are interconnected to cope with Cr(VI) toxicity. In a previous study we demonstrated that the aquatic fern *S. minima* growing in presence of increasing concentrations of Cr(VI), was able to accumulate high levels of metal in their floating and submerged leaves. Accumulation of metal was related to concentration of Cr(VI) in the treatment solution and was much higher in submerged than in floating leaves (Prado et al., 2010). When *Salvinia* plants grown in presence of increasing Cr(VI) concentrations a large increase of AP_{cap} was observed in both leaves (Table 1). However, in Cr-exposed leaves there were no increases of hexoses. This fact could agree with a previously reported hypothesis which considers that AP through an AOX activity acts to burn the excess of soluble carbohydrates (Millenaar et al., 2002). Our data, however, also showed a strongly increased content of sucrose in Cr-treated leaves (Table 2). Although this result could disagree with Millenaar's hypothesis, it has been reported that under stress conditions, increased AP seems to be accompanied by a stress-induced synthesis of sucrose, which may act as effective sink for the excess of ATP produced by cytochrome pathway (CP) (Solomos and Laties, 1975). In agreement with this assumption, our results did not show a negative effect of Cr(VI) on CP_{cap} in floating and submerged leaves, and even it was increased in Cr-treated leaves compared with Cr-untreated ones. Based on these findings, we assume that AP could have a key role in regulating intracellular concentration of soluble carbohydrates and redirectioning of carbon metabolism to

enhance cellular defence against Cr(VI). In fact, stimulation of AP would allow consumption of the excess of carbon to correct imbalance between carbohydrate supply and demand, thus controlling both anabolism and allocation of the fixed carbon (Sieger et al., 2005). In agreement with this assumption, values of the AP_{cap}/T_{resp} ratio revealed an increasing relative contribution of the alternative respiration to total respiration rate in Cr-treated leaves. Since sucrose is the link between assimilated photosynthetic carbon in the chloroplast and metabolic carbon consumption distant in space and time from it, understanding how Cr(VI) and AP activity can affect sucrose metabolism at cell level becomes very important to elucidate tolerance mechanism to Cr operating in leaves of *Salvinia*. Sucrose metabolism is controlled mainly by three enzyme activities: sucrose synthase (SS), invertase (I) and sucrose phosphate synthase (SPS); the first two being responsible of hydrolysis and the latter of synthesis. Hydrolysis of sucrose catalyzed by invertase has a higher ATP cost than hydrolysis catalyzed by SS. Invertase-derivative products requires two molecules of ATP to produce two hexose-phosphates, while SS-derivative products requires one ATP to give one hexose-phosphate and UDPGlc (Geigenberger, 2003). Since, increased AP leads to decreased ATP generation, it is possible to think that in our study, SS could be play the main role in sucrose metabolism of Cr-stressed leaves. In this context, under Cr(VI) exposure SS activity increased whereas soluble acid invertase activity (AI) decreased in both leaves. Further, glucose content was decreased under metal treatment, while fructose level tended to increase when compared with Cr-untreated leaves. Supporting our assumption on a most important role of SS in Cr-treated leaves, has been communicated that decreased glucose and increased fructose, reflects a lowered invertase/SS ratio that is considered an adaptive mechanism to save cellular energy (Shi et al., 2008). On the other hand, sucrose hydrolysis catalyzed by SS conserves energy binding of the glycosidic bond in resulting UDPGlc, which acts as substrate for the synthesis of cellulose and callose, or can enter into the glycolysis via combined action of UGPase and phosphoglucomutase (Winter and Huber, 2000). SS activity has also been correlated with starch synthesis and overall sink strength (Déjardin et al., 1997). In this sense, it was hypothesized that SS through the regulation of glycolytic intermediate pool, and AP through modulation of AOX activity by pyruvate, can adjust the accumulation of starch in rhythm with sink growth capacity (Gandin et al., 2009). Our data did not show increase of starch content in Cr-treated leaves, and even it decreased in submerged leaves (Table 2). Hence, could be assumed that synthesis and mobilization of starch in Cr-treated leaves involves other enzyme activities. SPS also plays an important role in carbohydrate metabolism due to it controls both sucrose synthesis and carbon allocation between sucrose and starch in response to changes in demand of sucrose imposed by stress conditions (Geigenberger et al., 1999). Agreeing with Geigenberger's assumptions, Cr(VI) affected differentially SPS activity in both leaf types, being enhanced in floating leaves and reduced in submerged ones. Overall, according with obtained results we assume that different metabolic events related to anabolism/catabolism of sucrose can be occurring in photosynthetic (floating leaves) and non-photosynthetic (submerged leaves) tissues of *S. minima* exposed to Cr(VI).

As far as it is known, exact role of R_{resp} was not clarified yet, but there are some evidences linking it to drought tolerance (Shugaeva et al., 2007). Moreover, it has also been involved in channelization of carbon skeletons towards secondary metabolism to enhance the synthesis of protective metabolites i.e. phenolics under mineral deficiency (Rakhmankulova et al., 2003). In agreement with these findings, our results showed significant increases of R_{resp} and SP in Cr-treated leaves of *S. minima*. Phenolics have been recognized as efficient antioxidants against heavy metal-induced oxidative stress due to their abilities to act as metal chelators (Psotová et al., 2003) and ROS scavengers through their capacity to donate electrons or hydrogen atoms (Michalak, 2006). However, phenolics can also participate in scavenging of ROS indirectly by coupling to a soluble peroxidase activity (Yamasaki et al., 1997). In agreement with this last finding, our results also showed an increase activity of soluble G-POD activity under Cr(VI) treatment. Interestingly, when analysing the content of SP in Cr-treated submerged leaves, a significant decrease was observed; while both R_{resp} and G-POD were enhanced. These apparently contradictory results could indicate no a reduced phenolic synthesis, but rather their polymerization, and then it can be correlated with highest content of polymerized phenolics i.e. lignin localized in cell walls of Cr-treated submerged leaves. Supporting our findings has been suggested a possible participation of R_{resp} in the synthesis of lignin through a peroxidase-catalyzed phenylpropanoid polymerization linked to a R_{resp}-induced production of H_2O_2 (Johnson-Flanagan and Owens, 1985). Moreover, G-POD activity was reported as key enzyme in the lignification process (Polle et al., 1994). On the other hand, polymerized phenolics are not extracted with ethanol 96% that is the solvent used for extraction of soluble phenolics, and then less soluble phenolics are estimated. Additionally, polymerized phenolics are undetected using standard colorimetric methods (Dai and Mumper, 2010). Likewise, highest accumulation of Cr(VI) found in submerged leaves was coincident with a previous report, which demonstrated that the main mechanism to accumulate Cr, Cd, Pb and Hg in epidermal glands of waterlily, an aquatic macrophyte, is the binding of metal to cell wall-polymerized phenols (Lavid et al., 2001). AP through AOX activity contributes to limit mitochondrial production of ROS, and thus constitutes an additional antioxidative mechanism against oxidative stress (Maxwell et al., 1999). Indeed, increased activity of AP could help in maintaining reduction of the respiratory chain components at an appropriate level and then diminish the generation of H_2O_2 (Popov et al., 1997). In this condition it is possible to maintain the production of ROS in balance with levels of low molecular weight antioxidants and antioxidative enzymes. Because of it has been suggested that AP plays a key role in counteracting deleterious short-term metabolic fluctuations, especially under stress conditions (Rasmusson et al., 2009), it could be assumed that increased AP also plays a critical role in reprogramming the metabolism of cells of Cr-stressed *Salvinia* leaves. Based on these assumptions, results reported in the present study show a metabolic interconnectivity among mitochondrial AP_{cap}, R_{resp}, sucrose, G-POD activity and soluble phenolics to optimize detoxification mechanism of Cr(VI) in leaves of *S. minima* plants. Moreover, data of this paper could contribute to clarify the residual respiration conundrum. However, mechanistic details underlying this metabolic adjustment process requires further investigation and represents an area of future research interest.

5. Conclusions

In conclusion, enhanced accumulation of sucrose and phenylpropanoid-derivatives in floating and submerged leaves of *S. minima* plants exposed to Cr(VI) seems to be positively associated with increases of AP_{cap}, R_{resp} and G-POD activity. Our results demonstrated that under Cr(VI) stress leaves switch the sucrose catabolism to energy conserving SS pathway rather than to less energy conserving AI pathway. This metabolic adaptation would be beneficial to *Salvinia* by reducing ATP consumption and improving performance of the carbohydrate metabolism, to provide more carbon skeleton to increase the synthesis of protective phenolic compounds. According to current knowledge, this work could represent the first communication about involvement of R_{resp} to support the tolerance of *S. minima* against toxic effects of Cr(VI).

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